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TITLE: Restoration of Immune Surveillance in Lung Cancer by Natural Killer Cells

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14. ABSTRACT

The goal of this application is to investigate how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. We have encountered problems with nicotine instability and work is still in progress to identify conditions to probe the effect of nicotine on NK cells. However, nicotine instability is not an issue in vivo and we have collected blood samples from tobacco smokers, e-cigarette users and past smokers to compare with never smokers for NK function. In terms of anti-miR183 therapeutics, we found that PLGA nanoparticles are readily taken up by NK cells but do not enter the lysosome, thus are not degraded within the NK cell. Nanoparticles will next be loaded with anti-sense miR183 to confirm that NK cell preloaded with anti-miR183 will resist TGFb suppression.

15. SUBJECT TERMS

Nothing listed

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1. **INTRODUCTION:**

Immune escape has emerged as one of the hallmarks of cancer and conquering this barrier is critical to early resistance against cancer. However, its mechanism remains obscure, especially related to natural killer (NK) cells. The goal of this application is to uncover how a microRNA, namely miR183, can disrupt the expression of a critical molecule, DAP12, that controls tumoricidal function in human Natural Killer (NK) Cells and to understand how nicotine, contained in tobacco smoke, utilizes this mechanism to abort immunity against lung cancer. In addition, we seek to explore the viability of targeting miR183 to restore NK cells as a new form of immunotherapy for early stage lung cancer. The specific aims are 1) to decipher the role of miR183 in nicotine-directed inhibition of human NK cell function, 2) to examine if the use of tobacco or nicotine-containing e-cigarettes induces miR183 expression and disrupt NK function, 3) to determine if targeting miR183 can serve as an immunotherapeutic modality for treating early stage lung cancer. These aims will be achieved through (i) analysis of molecular basis for nicotine control of the miR183 promoter, (ii) analysis of heavy tobacco smokers and e-cigarette smokers as well as former heavy smokers for loss of NK function, and (iii) analysis of a human xenograft *Nod-scid-IL2Rg*$^{-/-}$ (NSG) mouse model for the ability of human NK cells to respond to NK-directed nanoparticles bearing antagomiRs against miR183 to provoke human lung tumor regression in vivo. Such approaches will yield new insight into the pathogenic role of miR183 in nicotine-derived NK cell suppression and define a new miR-based immunotherapeutic strategy to treat lung cancer.

KEYWORDS: Natural Killer Cell, NK receptors, tumor microenvironment, Transforming Growth Factor-beta, nicotine, tobacco smokers, e-cigarette-users, lung cancer, microRNA-183, DAP12, NKp44, NKp46, nanoparticle-based immunotherapy

2. **ACCOMPLISHMENTS:**

What were the major goals of the project?

Lung cancer mortality remains a leading therapeutic issue because of the heterogeneity of cell types and the diversity of genetic changes involved. New drugs offer an improved overall survival to only a small subset of patients and the majority of lung cancer patients can only be treated with palliative chemotherapy. Immune surveillance, on the other hand, can be effective against any form of lung cancer, if mobilized in the early stages of disease. Natural killer (NK) cells are particularly effective as the first line of innate immunity against cancer. However, we recently made the seminal finding that NK cells in the lung tumor microenvironment are significantly at a disadvantage because of loss of DAP12, a critical adaptor protein that anchors activating NK receptors on the cell surface to recognize tumor cells. This loss is caused by transforming growth factor beta (TGFb) produced by tumor cells that can induce microRNA (miR)-183 in infiltrating NK cells to downregulate DAP12 expression. In addition to TGFb, tobacco smoking has long been established to cause lung cancer, and nicotine contained in tobacco smoke is reported to be immunosuppressive. With the discovery of miR183 as a critical NK cell regulator, the goals of the project are to investigate (i) if nicotine associated with tobacco smoking and lung cancer induces the miR183/DAP12 circuit to suppress NK function, (ii) if use of tobacco or e-cigarettes induce miR183 an disrupt NK function, and (iii) if miR183 blockade can be targeted to treat early stage lung cancer by reactivating NK cell function.

What was accomplished under these goals?

Aim 1: To examine the role of miR183 in nicotine inhibition of human NK cell function.
Tobacco smoking has been associated with immune suppression and, based on our preliminary data that Nicotine and the nicotine metabolite, 4-(methyl-nitrosamine)-1(3-pyridyl)-1 butanone (NNK), reduced intracellular DAP12, surface NKp44, and lysis of A549 lung tumor cells by NK cells in vitro, we had proposed to extensively analyze the mechanism of action. To our disappointment, these observations have not panned out. We have conducted all the proposed experiments with no significant results. We have tested a number of new lots of nicotine and each lot was tested on different normal donor NK cells but, so far, we have not seen any reduction in DAP12 or NKp44 levels. We also evaluated the metabolite, NNK, as well as e-nicotine but neither had any effect on NK activation markers, whether they were measured by flow cytometry, western blotting, qPCR. In all experiments, transforming growth factor beta (TGFb), was included as a positive control, indicating that the assay systems were functional. In terms of tumoricidal function, neither NNK nor e-Nicotine could alter NK lysis of A549 lung tumor cells or lysis of a variety of other tumor cells including K562, PSCS, Panc-1 and Mia-Paca. Even when the treatment with NNK or e-nicotine was extended from 24 h to 5 days, no modulation of NK markers or lytic function was observed. NK cells are known to express nicotine receptors but blockade of these receptors by bungarotoxin (competitively binds to α7/α9 nicotine receptors) or anti-α7/β4 antibody (block α7/β4 nicotine receptors) did not alter the level of NK markers.

In conclusion, we were unable to demonstrate that nicotine had any effect on NK function.

Aim 2. To verify that use of tobacco or e-cigarettes can induce miR183 and disrupt NK function.
DOD Human Research Protection Office of our IRB to evaluate the effect of tobacco smoking or e-cigarette use on NK cells was approved about 4 months after the initiation of the grant and we began to collect blood samples and, of the 20 samples for each group proposed to be collected, we have so far processed peripheral blood mononuclear cells from 15 current smokers, 10-cigarette users, 8 past smokers and 8 never smokers. Collection still continues. We have frozen all the materials in aliquots and tested the collected samples together in one experiment for NK markers, in order to minimize day-to-day technical variability.

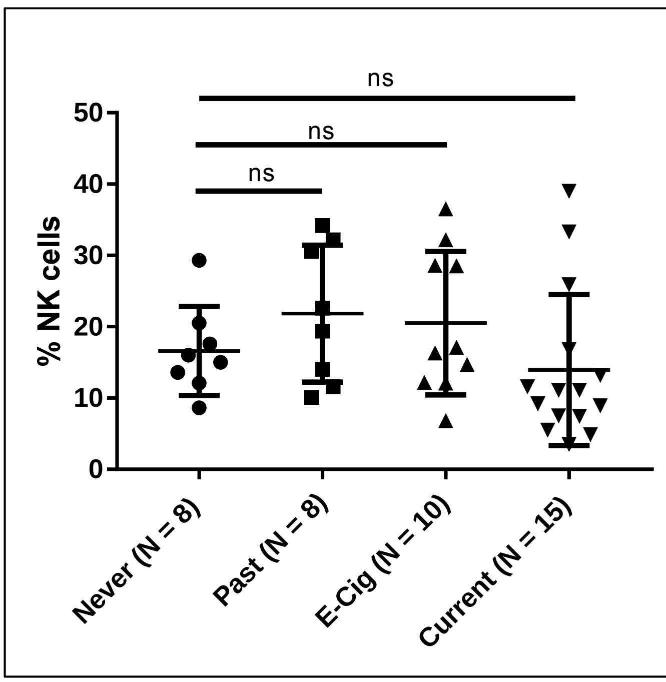

Fig. 1. Percentage of peripheral blood NK cells in never, past, e-cigarette and current tobacco smokers.

We found that the percentage of NK cells in the blood was not significantly different among the 4 groups, although there was a trend to lesser NK cells in current smokers **(Fig.1).** The mean NK percentage for current smokers was 13.95+2.7, e-cigarette users was 20.5%+3.2, past smokers was 21.8+3.4 and never smokers was 16.6+2.2. We also tested whether DAP12, NKp44 or CD16 was reduced in the NK cells. There was a wide range of % DAP12+ NK cells with no significant difference among the groups **(Fig.2A).** The level of DAP12 expression in the NK cells, as measured by mean fluorescence intensity was also not significantly different among the groups

(Fig. 2D). Of note is that the percentages of NKp44 expressing NK cells are very low in all groups, and the mean fluorescence intensity of NKp44 is unchanged by tobacco or e-cigarette smoking **(Fig. 2B,E).** NKp44 is an activation marker, inducible by IL2, and it appears that NK cells freshly obtained from blood from all groups remain naïve and unstimulated by tobacco or e-cigarette. We also included CD16, the FcR for IgG, on NK cells **(Fig 2C,F),** and found that the percentage of CD16+ NK cells is slightly lower in current smokers (34.6±4.7) and e-cigarette users (30.1±7.8) as compared to past smokers (44.3±7.2) and never smokers (43.3±4.4).

Our results, taken together, suggest a trend in reduced percentage of NK cells in current smokers, especially CD16+NK cells, where CD16 FcR is critical for antibody-dependent cell cytotoxicity against microbially-infected cells. We therefore intend to complete collection of all 20 samples per group to have a more statistically relevant comparison among the groups.

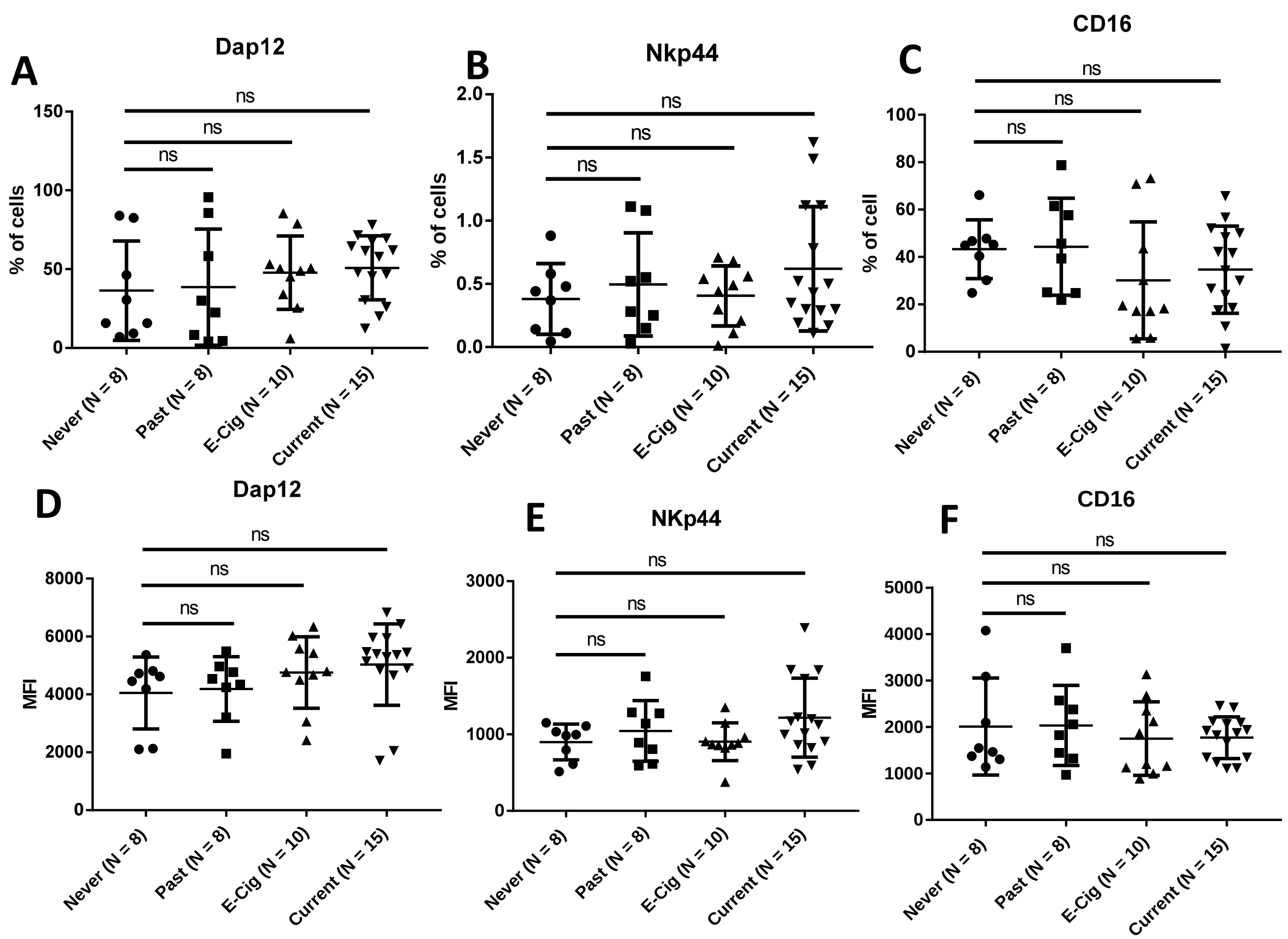

Fig. 2. Percentages and mean fluorescence intensity of DAP12, NKp44 or CD16 expressing NK cells in never, past, e-cigarete, and tobacco smokers.

<u>Aim 3. To construct stable anti-sense miR and nanoparticles targeted to human NK cells</u>.
This aim intends to restore NK function against tumor cells by targeting miR183 that is induced in NK cells by TGFb in the tumor microenvironment. In order to answer if nanoparticle-based

anti-miR183 can be an effective therapeutic strategy, an in vivo xenograft model was developed to accept and maintain human NK cells.

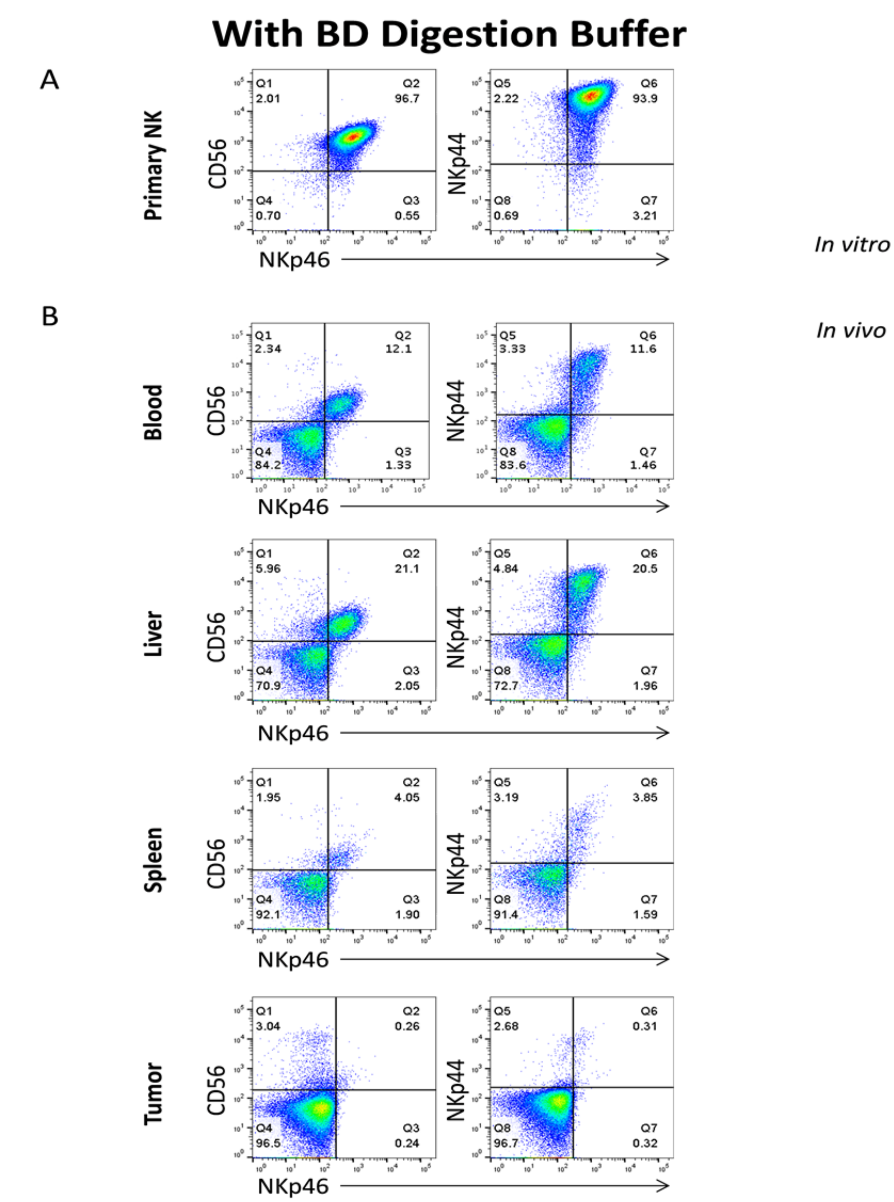

Figure 3. Flow cytometric analysis of primary human NK cells cultured in vitro (A) and recovered in vivo in different organs after intravenous injection of 1 x107 NK cells into NSG mice bearing human A549 lung tumors (B). Mice were subcutaneously inoculated with $5x10^5$ A549-Luciferase on the back. When the tumors reached a detectable size, mice were injected via tail vein with total of 200ul of 10^7 NK cells, supplemented with 500U/ml IL-2 and 50ng/ml IL-15. Mice then were sacrificed at day 3 and lymphocytes were isolated from tumors and different tissues using Ficoll density gradient centrifugation. The isolated lymphocytes were analyzed for CD56+NKp46+ or NKp44+NKp46+ NK cells. These NK cells are readily detected in blood, liver, and spleen but not in the tumor bed.

We have optimized conditions to grow and maintain human NK cells both in vitro and in vivo in NSG mice. Culture of NK cells with IL15 for 24h prior to in vivo tail vein injection into mice and the addition of IL15 to the injection medium significantly improved the detection of NK cells in tissues of mice bearing human A549 lung tumors. However, we faced the problem of recovering few NK cells (0,6%) in the tumors of mice, although significant numbers could be seen in the blood (22.1%), liver (22.1%) and spleen (8.47%), as seen in a representative experiment. We surmised that the reason of recovering few NK cells in the tumor bed was due to harsh conditions to isolate human NK cells. We found that the commercial digestion buffer to dissolve the tumor causes cell death of both tumor cells and infiltrating NK cells. Viability of tumor cells ranges from 50% to 60%, and because the injected NK cells form a very small percentage of the cells within the tumor tissue, they could easily be lost. We attempted to use other commercial digestion buffers and finally found one from BD Life Sciences that provided us with

94-97% viability (Fig. 3A). With this product, we have resumed to examine the trafficking of human NK cells into organs of A549-tumor bearing NSG mice.

Using either CD56/NKp46 or NKp44/NKp46 as specific NK markers and the BD Digestion Buffer, we found that NK cells still did not appear to enter the tumor but can be detected in the blood, liver, and spleen **(Fig. 3).** To determine if this is a phenomenon related to the tumor cell line used, we next tested NK cell infiltration into either A549 tumor-bearing mice or H1299 lung tumor-bearing mice **(Fig. 4A,B).** Gating on CD45+ human lymphocytes, the same results were obtained with both cell lines in vivo, using NKp46/NKp44 or NKp46/NKG2D markers to identify NK cells.

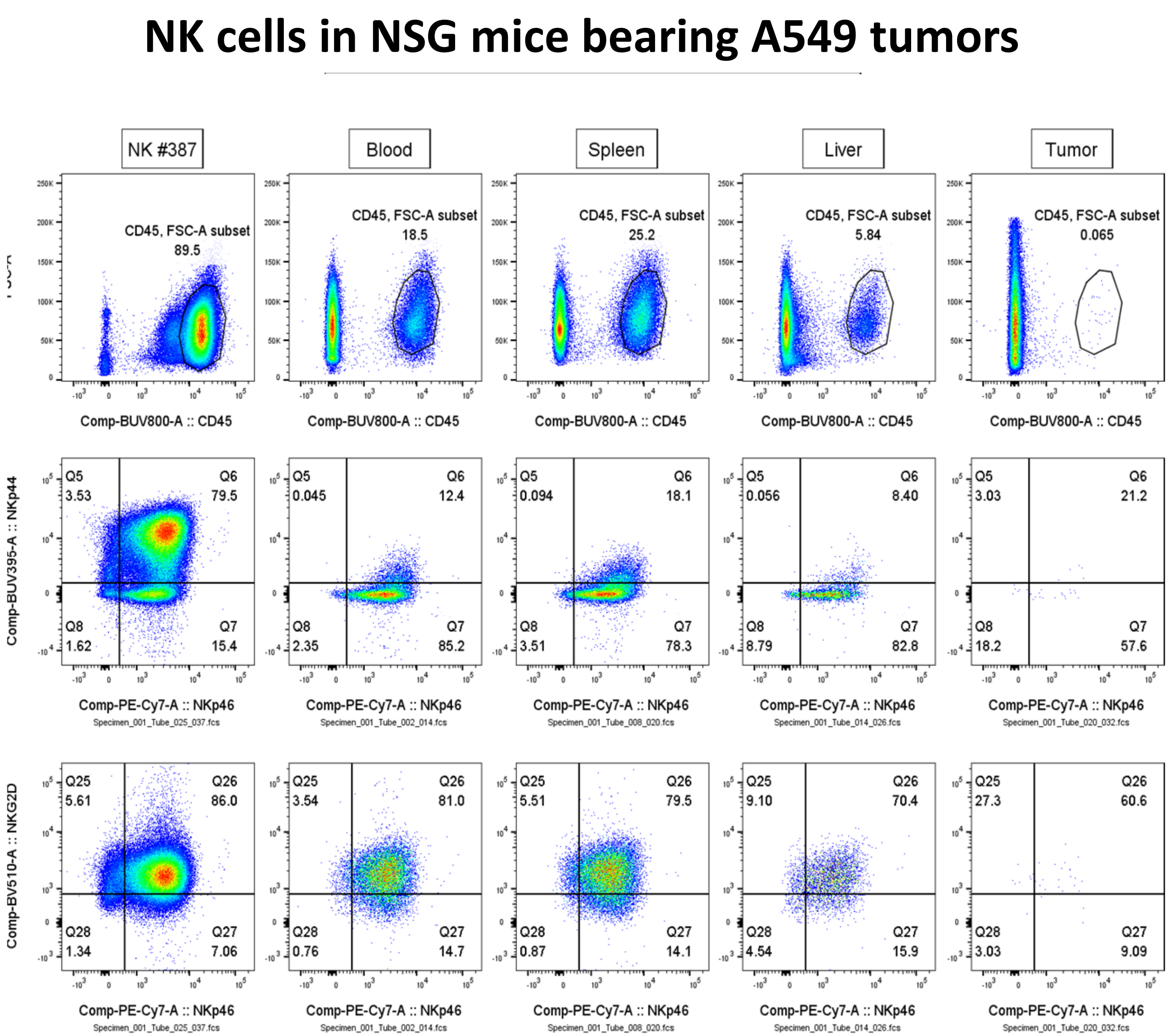

Fig. 4A. Percentages of CD45+ human lymphocytes in different organs of A549 lung tumor bearing NSG mice. Gating on CD45+ human lymphocytes, the percentages of NKp46+NKp44+ cells or NKp46+NKG2D+ cells were assessed in the various organs. NK cells are captured in blood, spleen and liver but rarely in tumor tissue.

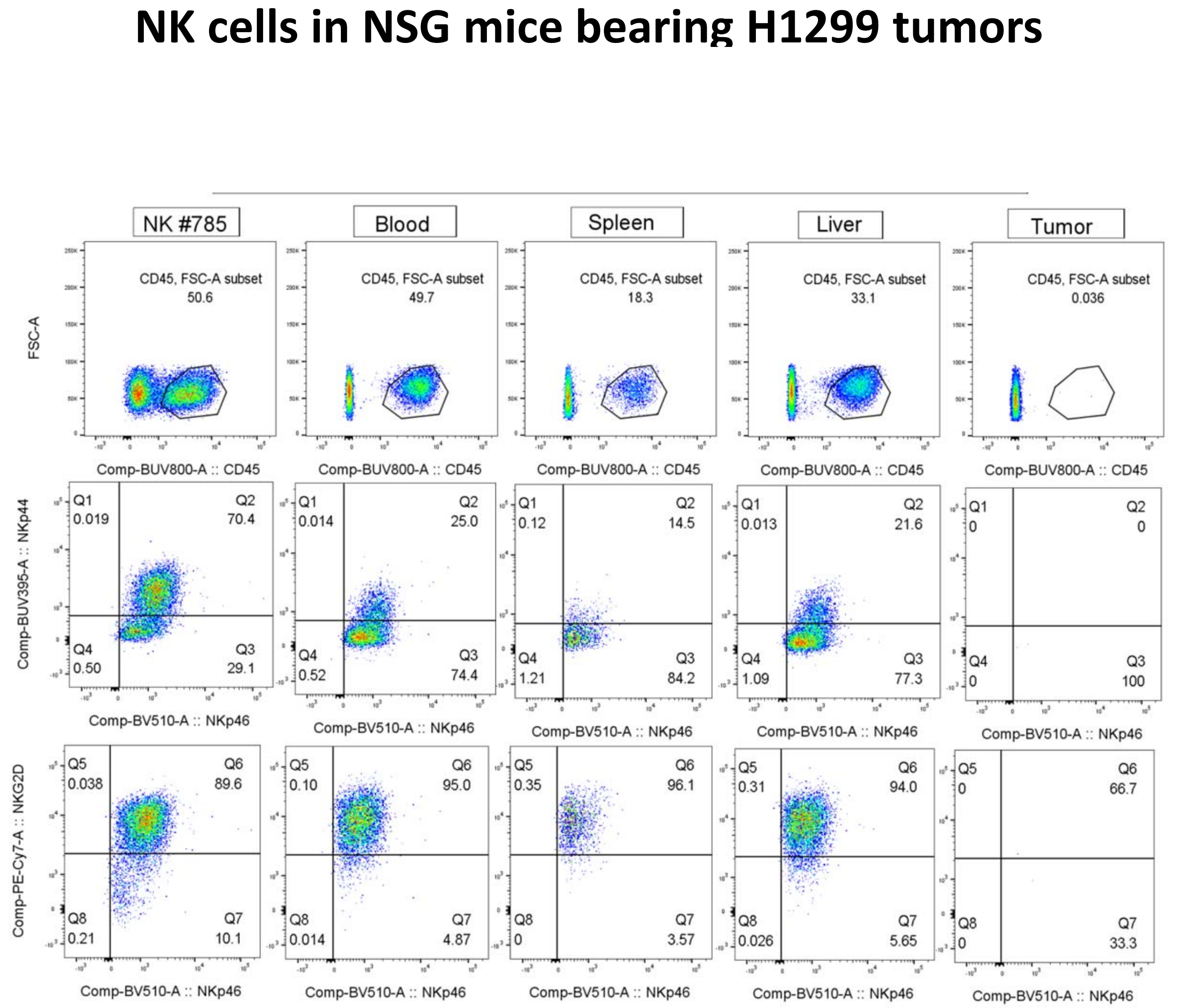

Fig. 4B. Percentages of CD45+ human lymphocytes in different organs of H1299 lung tumor bearing NSG mice. Gating on CD45+ human lymphocytes, the percentages of NKp46+NKp44+ cells or NKp46+NKG2D+ cells were assessed in the various organs.

The lack of significant detection of NK cells in tumor tissue could be due to loss of chemokine receptors in NK cells caused by the tumor microenvironment or due to lack of chemokines produced by the tumor cells to attract NK cells. The chemokine and chemokine receptor family is extensive but NK cells are known to express certain chemokine receptors, including CCR2. We have reported earlier that A549 tumor cells lack CCL2 which bind CCR2, and thus may not attract NK cells. We are attempting to define if CCL2/CCR2 or other chemokine/receptors are involved.

We have successfully encapsulated miRNA183 into PLGA nanoparticles (NP)s while maintaining miRNA integrity, with a loading efficiency of 25%. These NPs also have no untoward cytotoxic effects on NK cells in vitro. PLGA nanoparticles (NP) appear to be easily taken up by human NK cells in vitro and they are not targeted into lysosomes, thus indicating that the anti-sense miR183 cargo within PLGA NPs will not be degraded inside the NK cell.

In order to test the product in vitro prior to in vivo therapeutics in NSG mice, we first developed a 3D poly(ethylene glycol)-based hydrogel system for the study of NK cell-lung cancer cell interactions. In this system, NK cells migrated into hydrogels containing primary A549 cells but only at 24h after tumor implantation. The tumor cells were incubated in the hydrogels for 24 h (early stage) or 7 days(mature stage) prior to introduction of NK cells to mimic various stages of the tumor microenvironment. With increasing culture time, the A549 cancer cells remain viable and form cell aggregates/clusters in the gel. Interestingly, after culturing A549 cells for 7 days, NK cells show significantly lower migration into the A549 gels (Fig 4A). These finding recapitulate in vivo results showing that when NK cells are co-injected with cancer cells in mice, tumors do not form; however, when the cancer cells are injected first and NK cells 7 days later, the NK cells do not impact tumor development, and tumors do form. Therefore, we believe our in vitro model may recapitulate important features of the in vivo tumor microenvironment. Cytokine analysis for the 3D models revealed that NK cells are indeed less activated in the "mature" tumors than in "early stage" tumors, as determined by decreased levels of RANTES, a chemokine secreted by activated NK cells to recruit other immune cells (Fig 4B).

We plan to complete our characterization of this model system in vitro and compare the results with tumor xenografts in vivo, in order to validate that our in vitro system models in vivo mechanisms and is an effective system for evaluating immunotherapies in vitro.

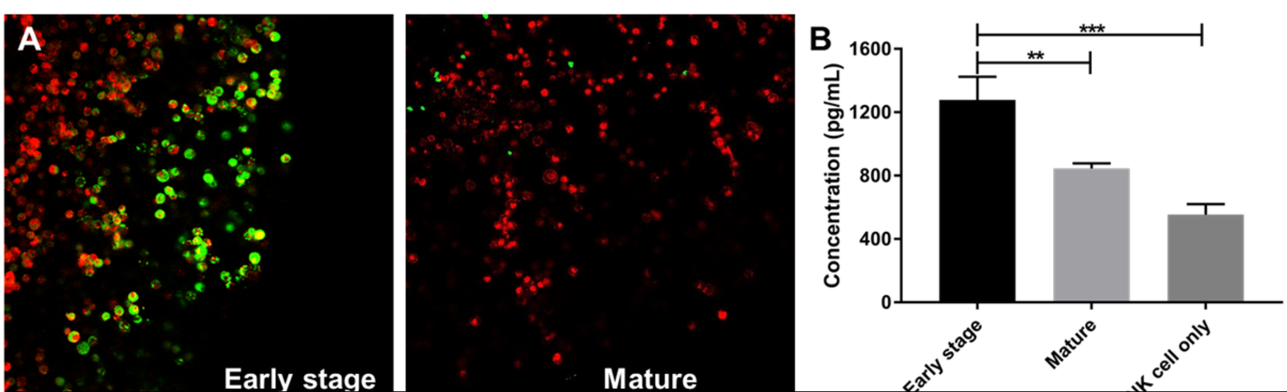

Figure 4. (A) Migration of NK cells into a 3D tumor model is impacted by the incubation period of the A549 lung cancer cells in the hydrogel. Red = cancer cells; yellow = NK cells. "Early stage" tumors refer to hydrogels in which A549 cells were cultures for 1 day, and "mature" tumors refer to hydrogels in which A549 cells were cultured for 7 days prior to the introduction of NK cells. There is decreased NK cell migration into mature tumors. (B) Secretion of RANTES by NK cells in coculture with "early stage" and "mature" tumor models compared to NK cells in isolated culture.

What opportunities for training and professional development has the project provided?
Nothing to report

How were the results disseminated to communities of interest? Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Aim 1: To examine the role of miR183 in nicotine inhibition of human NK cell function.

We continue to look for other sources of nicotine and are consulting colleagues who also are facing the same issues of nicotine instability to see if they have come up with solutions to stabilize the compound.

Aim 2. To verify that use of tobacco or e-cigarettes can induce miR183 and disrupt NK function.
We already have some trends observed where current smokers may have reduced percentages of NK cells in circulation compared to past smokers or never smokers. We will finish collecting the rest of the 20 samples per group from never smokers, heavy tobacco smokers, past smokers and e-cigarette users and then test for intracellular DAP12 and surface NKp44 by flow cytometry and for tumoricidal function by Cr-release assay, followed by data analysis. Till now, we have only evaluated flow cytometric analysis of NK markers such as DAP12 and NKp44. We plan to use saved aliquots of all the samples for analysis of miR183 expression by qPCR and for lytic function against lung tumor cells.

Aim 3. To construct stable anti-sense miR and nanoparticles targeted to human NK cells.

We will begin to examine if PLGA nanoparticles loaded with anti-sense miR183 can be effective in downregulating miR183 first in the luciferase HELA reporter construct model and then in the human NK cells themselves. We will thus optimize nanoparticle formulation for delivery of anti-miR183 in NK cells. Once optimized, we will then employ the anti-miR183-encapsulated nanoparticles for immunotherapy of lung cancer in NSG mice. We will first preload the nanoparticles into NK cells prior to their intravenous administration into NSG mice bearing A549-human lung tumors. If successful, we will then introduce the nanoparticles in free form given intravenously into tumor bearers that have already been administered NK cells.

3. **IMPACT:**

TGFb has long been known to be immunosuppressive but clinical trials with anti-TGFb or TGFb inhibitor in cancer patients have not produced notable successes, likely due to numerous off-target effects related to the pleiotrophic nature of TGFb with various physiological effects. Direct targeting of miR183 could avoid such issues to constitute a new molecular and precise strategy for lung cancer therapy. Most importantly, we have discovered a new immune checkpoint inhibitor, miR183, that disrupts NK function against cancer.

What was the impact on the development of the principal discipline(s) of the project?

To date, no attempts have been made to address microRNA therapeutics to correct tumor immunity against cancer and our project is of high impact in introducing precision medicine to control a new immune checkpoint inhibitor.

What was the impact on other disciplines? *Nothing to report*

What was the impact on technology transfer? *Nothing to report*

What was the impact on society beyond science and technology? Nothing to report

4. **CHANGES/PROBLEMS:** *Nothing to report*

Changes in approach and reasons for change: Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. *No changes*

Significant changes in use or care of human subjects. *No changes*

Significant changes in use or care of vertebrate animals. *No changes*

Significant changes in use of biohazards and/or select agents. *No changes*

5. **PRODUCTS:** *Nothing to report*

Website(s) or other Internet site(s): *Nothing to report*

Technologies or techniques: *Nothing to report*

Inventions, patent applications, and/or licenses: *Nothing to report*

Other Products: *Nothing to report*

6. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

What individuals have worked on the project?

Name:	*Julie Y. Djeu*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*2*
Contribution to Project:	*No change*
Funding Support:	*No change*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? *No change*

What other organizations were involved as partners? *Nothing to report*

7. **SPECIAL REPORTING REQUIREMENTS** *Nothing to report*

8. **APPENDICES:** *Nothing to report*